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THE CULTIVATION OF MALARIAL PLASMODIA (PLASMODIUM VIVAX AND PLASMODIUM FALCIPARUM) IN VITRO.*

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INTRODUCTION.

In November, 1911, one of us (Bass (1)) reported the successful cultivation of *Plasmodium vivax, Plasmodium malariæ,* and *Plasmodium falciparum in vitro.* Though the technique was not given in detail, it was stated that the incubation temperature must be 40° C. or higher, and that defibrinated or citrated human blood seemed to be the most favorable culture medium. Strict anaerobiosis seemed also to be necessary. Further experimentation along this line was interrupted by unavoidable circumstances until April, 1912, when the Tulane School of Tropical Medicine and Hygiene of the Medical Department of Tulane University equipped and sent a research party to the Canal Zone for the study of malaria.[1]

Our efforts were directed toward a critical experimental study of the factors involved in the cultivation of malarial plasmodia *in vitro,* with the hope of developing a technique that would yield more uniform results than had been obtained in the earlier work. The work has resulted in developing a technique by which at least one cycle of schizogony can be cultivated in practically every instance, and many if not an indefinite number of generations can be cultivated in most instances.

* These experiments comprise Study 31 of a series conducted under the direction of Dr. Creighton Wellman. Preliminary Report of the First Research Expedition to the Tropics from the Tulane School of Tropical Medicine and Hygiene. Received for publication, July 19, 1912.

[1] The expenses of this party were paid out of a fund contributed to the School for this purpose through the Dean of the Medical Department of Tulane University, by a person or persons whose name or names are unknown to us. Transportation for ourselves and apparatus was contributed by the United Fruit Company.

Though our prime object was the cultivation of malarial plasmodia we have observed other important facts relative to different phases of the subject of malaria. This communication deals only with the cultivation of malarial plasmodia and is not intended to be a full report of the work done and the observations made by this Commission.

HISTORICAL NOTES.

Laveran (2) reported malarial parasites to be visible in hanging drop preparations for as long a period as ten days. He was apparently not able to satisfy himself that any multiplication took place, nor, indeed, that the parasites remained alive. Coronado (3) claimed to have cultivated malarial plasmodia from infected water, but his work has not been confirmed. In the light of our experiments his claim is at once set aside by the fact that the parasite cannot live for any length of time in any non-isotonic fluid. Such fluids as water are absolutely destructive not only to the red blood cells which are necessary for the growth of the parasites, but also to the plasmodia themselves. Miller (4) reported also that he cultivated the parasites, but his experiments, which have been repeated, have not been confirmed. Sakharov, Rosenbach, Blumer, Hamburger, and Mitchel (5) kept malarial plasmodia alive for several days in leeches that had been allowed to draw the blood of malarial patients. In the instances of Hamburger's experiments, the æstivo-autumnal parasites developed motile pigment. In Blumer's case the parasites showed no ameboid movement. In both instances the parasites became peculiarly refractive and glistening in appearance. Our observation has led us to consider these forms as dead parasites. None of the beautiful segmentation and rosette formation, which are seen in all our successful cultures in forty-eight hours or less, were noted even in eight days. Their cultures, however successful, must be considered as having been accomplished *in vivo* instead of *in vitro*.

We fully appreciate the fact that our present technique is imperfect and includes steps which are more or less empirical. No doubt the present methods will appear very crude a few months hence. They will serve, however, as a basis for further development. Simple as the present technique is, months of patient work and innumerable experiments have been required for its development.

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TECHNIQUE.

Apparatus and Material.—The apparatus and material necessary for the cultivation of one generation only of parasites are as follows:

1. Syringe and needle. We use a twenty cubic centimeter all glass syringe with coarse needle. The latter is necessary to avoid strong capillary force and strong suction which so alter the host cells (or parasites), that the parasites often die in a short time. The large needle is also desirable to avoid leaking and bubbling of air into the syringe and to facilitate taking blood into and expelling it from the syringe.

2. Defibrinating tube. Tubes one inch in diameter and of whatever length will be accommodated by the centrifuge (if a centrifuge is to be used) are appropriate. The tube is plugged with a cotton plug having a glass rod running through it and extending to the bottom. We have used with equal satisfaction a glass tube in place of the rod. To this is attached the needle by means of a short piece of rubber tubing. The blood is collected directly into the defibrinating tube by sticking the needle into an infected vein.

3. Culture tubes one half by five inches.

4. Graduated pipette, one cubic centimeter, graduated in hundredths.

5. Dextrose (Merck's), 50 per cent. solution in water.

6. Capillary pipettes and rubber bulb. Three to four sixteenths of an inch glass tubing is the proper size from which to make these pipettes. The capillary tube should be rather large. Fine capillaries are destructive to malarial plasmodia. The large end of these pipettes should be plugged with cotton before sterilizing.

7. Incubator regulated to a temperature of 40° C.

The following additional apparatus will be required if more than one generation of parasites is to be grown, or if it is desired to avoid the dead parasites which are present in the deeper layers of cultures containing a thick column of cells.

8. Centrifuge. Speed, 800 to 2,000 revolutions per minute.

9. Culture tubes one half by five inches, with flat bottom or with disk of pure white filter paper supported one half inch from the bottom of the tube by a piece of glass tubing. The latter should be

cut straight across and fairly smooth on the upper end and should fit the culture tube closely.

10. Plain pipettes, capacity five to twenty cubic centimeters, with rubber tube two feet long, mouth piece, and pinch cock. These pipettes are plugged before sterilizing and are used but once on account of the difficulty of cleaning them.

Technique for Cultivating One Generation Only.—Blood is collected from the patient's vein at the bend of the elbow. If drawn with the syringe it is expelled directly into the defibrinating tube. The latter should be tilted to one side and care should be taken to avoid unnecessary exposure of the blood to the air. In either case, one tenth of a cubic centimeter of the 50 per cent. solution of dextrose for each ten cubic centimeters of blood to be taken is placed in the defibrinating tube before the blood is drawn. Defibrination is effected by gently stirring or whipping with the rod or tube which extends through the cotton plug. The whipping in of air, causing bubbles, must be avoided. The plug and rod may now be replaced by a plug from another tube of the same size.

The defibrinated dextrose blood may be transferred to other tubes or incubated in the original tube. In any event the column of blood must be one to two inches deep. This gives a column of serum one half to one inch deep above the cells and parasites when the latter have settled. Supernatant serum more than one inch deep has no advantage. When this is less than one half of an inch deep the parasites often die before segmentation occurs. We have occasionally seen perfect segmentation, however, under one fourth of an inch of serum.

The parasites live and develop at the top of the column of precipitated cells in a layer varying in thickness from one fiftieth to one twentieth of an inch. All parasites beneath this layer die in from two to twenty hours; the time is dependent upon factors which we have not determined. Some grow considerably before they die and we have occasionally seen small rings attain over half the full adult size under these circumstances. When parasites die in these deep cells the central clear space in the small ring closes, or if the parasite is older the irregular projections, pseudopodia, are withdrawn. If pigment is present, it loses its motility. The protoplasm

gradually shrinks, losing its staining reaction, and finally only the nuclear chromatin granule remains, and this also stains poorly. In the instance of very small parasites often the red blood cell shows little or no evidence of the previous presence of the parasite.

The parasites in the thin layer at the top of the column of cells develop and may be examined at any time by drawing a small quantity of cells from this layer by means of a capillary pipette. Some considerable practice is required in order to do this without drawing cells and dead parasites from just below this layer. The pipette may be passed through a flame to sterilize it on the outside, but it must be allowed to cool thoroughly before it is used, since, in our experience, a temperature of 45° to 50° C. kills the parasites in a very short time.

Such a temperature also alters the red blood cells in some way so that they are rendered permeable to the surrounding serum, and this effect is destructive to the parasites. Great care must be taken in handling tubes containing cultures to keep them in the upright position. Tilting to the side results in burying and killing the living parasites in the thin layer at the top of the cells which have settled to the bottom of the tube.

Technique for Cultivating More than One Generation.—If more than one generation of plasmodia is to be cultivated, it is necessary to remove the leucocytes when the culture is made in order to avoid destruction of the parasites by them at the time of segmentation. The infected blood from the patient is centrifugalized sufficiently to force the leucocytes to the surface of the cells. The length of time necessary to centrifugalize varies with the speed, length of arm, etc., of the particular centrifuge used and should be determined by experiment. Unnecessary centrifugalization should be avoided.

The supernatant serum is drawn off and put in culture tubes. The column of serum in each should be one half to one inch deep. Cells and plasmodia are carefully drawn from about the middle of the centrifugalized cells and planted at the bottom of the serum in the culture tubes. Flat bottom tubes are an advantage. One to two tenths of a cubic centimeter of cells in a half inch tube make the thickest layer in which it is possible to get a homogeneous growth of parasites. We have been able to secure growth of all the parasites

in about twice this quantity of cells in a half inch tube by employing tubes with a paper shelf suspended in them. They are filled with serum to at least half an inch above the level of the support for the paper disk. The disk is then carefully tucked in place, after which the cells are placed on it.

We have obtained even more satisfactory results with tubes filled one half inch deep, or more, with freshly prepared human plasma. These are conveniently prepared by placing one half to one inch of blood, immediately after it is drawn, in culture tubes and centrifugalizing to throw the cells to the bottom before coagulation takes place. By continuing centrifugalization until after coagulation occurs, a flatter surface is secured for the plasma than if it is discontinued before this time. In such plasma prepared tubes one half inch or more of serum is placed and then cells and parasites from the centrifugalized and defibrinated dextrose blood are carefully distributed over the surface of the plasma. We have sometimes obtained in these tubes a layer of live parasites approximately one tenth of an inch thick.

Parasites in such leucocyte-free cultures develop, segment, and most of the merozoites enter new red blood cells. These young parasites develop in the same manner as the first generation and sometimes reach the stage of segmentation. In fact we have in one instance observed the development of three successive generations in such a culture. More often, however, the parasites begin to die out after the first segmentation and especially after the second. We have not been able to determine exactly the cause of this. In order to perpetuate the culture it is necessary to transfer a portion of the cells and parasites to a recently prepared tube containing fresh cells and serum. It is convenient to place the fresh serum in the culture tube and to take up in a large capillary pipette a portion of the cells and parasites of the culture and then about five times the amount of fresh cells. These are mixed in the pipette with air excluded and then carefully spread on the surface of the plasma, paper shelf, or bottom of the tube, according to the particular kind of culture tube used. The transplantation should be done within four or five hours of the time of maximum segmentation and therefore approximately every forty-eight hours for tertian and æstivo-autumnal parasites.

DISCUSSION.

In successful cultures the asexual parasites grow, segment, and form rosettes, which burst and give rise to merozoites, many of which enter new red blood cells in exactly the same manner as they do in the body of man. The young parasites increase in size slowly during the first twenty-four hours, after which they grow much more rapidly. Segmentation begins in about thirty-six hours from the smallest ring-form stage. The chromatin granule first divides into two parts, and in segmenting cultures parasites containing any number up to about twenty segments can be found. The largest number is seen in tertian plasmodia.

Most of our experiments have been with the first and second generations. We have carried one culture of æstivo-autumnal parasites through four generations by the method given above and believe that with sufficient care they can be carried through an indefinite number of generations.

We have cultivated one or more generations of æstivo-autumnal parasites from each of twenty-nine different patients. We have also cultivated the tertian parasite six times. Only one case of quartan was tested, but the parasites failed to grow, as we now believe, on account of certain errors in our earlier technique. It is to be remembered that one of us (Bass (1)) obtained growth of the quartan parasite in his earlier work.

Æstivo-autumnal parasites seem to be more resistant to unfavorable conditions in the cultures and to slight errors in the technique than the tertian. When everything is exactly right, however, the latter grow equally as well as the other variety.

In cultures containing gametes (tertian) we have noticed that the enveloping red blood cell is more resistant to unfavorable conditions and retains its color for a much longer time than cells containing schizonts. Many of these parasites increase in size, and they usually live several hours or days after the asexual parasites have died out. We have not made any systematic study of the sexual parasites, but in many instances we observed what we interpreted as an example of parthenogenesis, and in one instance what we believe was a four day old zygote. These observations indicate the possibility of cultivation of the sexual cycle.

Serum from different individuals is not equally serviceable for the cultivation of malarial plasmodia. The parasites failed to grow in the presence of some of the sera we experimented with. We have cultivated both tertian and æstivo-autumnal plasmodia in the presence of Locke's fluid, minus calcium chlorid, and in the presence of different ascitic fluids, of which one had been drawn nearly two months. Dextrose was always required and the growth was never so good as when human serum was used.

The parasites have been grown in the presence of red blood cells only. We have not seen any evidence that they can be grown independently of these cells. As already stated, they cannot live for even a few minutes free in the serum. Serum inactivated of its complement is less destructive, but the plasmodia cannot live in it for any considerable length of time. When cultures are prepared according to the technique described for the cultivation of one generation only, the leucocytes migrate more or less toward the surface and soon become actually concentrated in the layer in which the parasites grow. Leucocytes do not phagocytize parasites as long as they are inside of the red blood cells, but as soon as segmentation takes place and the capsule of the red cells ruptures, liberating merozoites, the latter are promptly engulfed. In fact, it is not uncommon to see a leucocyte that has phagocytized one or more full rosettes. This probably occurs after the parasite has digested the capsule or otherwise made a small opening through it, whereby it is converted into a foreign body which the leucocytes try to remove. Dead parasites in red blood cells are also phagocytized, providing the enveloping cell substance is sufficiently permeable. Parasites phagocytized are soon killed and finally digested. As a result of this phagocytosis few if any parasites in such a culture escape to develop a second generation.

Other carbohydrates which we tried are saccharose, lactose, galactose, dextrin, mannite, and maltose. Maltose alone seems to be equal to dextrose for this purpose. We are quite certain that the dextrose exercises other important influences than simply the control of oxygen. Blood taken and kept under the most perfect anaerobic conditions will not serve for cultivation of parasites unless dextrose or maltose is added.

We have noted also that blood drawn within one or two hours

after the patient had eaten a full meal was much more serviceable for culture media than if drawn after long fasting. In fact we failed to grow tertian parasites in blood after the patient had been fasting, even though the usual quantity of dextrose was added. This fact is in keeping with the clinical observation that the parasites will often disappear from the blood and the paroxysms cease if the patient is put to bed and is given a purgative and light diet. It is possible that violent exertion and chilling of the surface, both of which often precipitate a malarial chill in patients who would otherwise have missed the paroxysm, act by increasing the dextrose circulating in the blood to meet the requirements of combustion for the production of energy or heat. Or it may be that the dextrose, by reason of its very large molecule, influences the permeability of the red blood cell to substances in the serum which are very destructive to malarial plasmodia. An asexual malarial parasite regardless of its size or age is killed by exposure to normal serum, malarial patient's serum, or any modification of these that we have been able to make. This is particularly noticeable at the time of segmentation of a crop of parasites. If at this time the growing parasites are shaken up or mixed with serum it will be found that even within a few minutes all free merozoites and rosettes whose red blood capsules have become permeable to the serum have died and stain faintly.

The most rapid growth is obtained at a temperature of 40° to 41° C. In fact, æstivo-autumnal parasites will develop from the smallest rings and segment in about thirty hours at a temperature of 41° C., while they require forty-eight hours in the body of man. Parasites will usually develop, but more slowly, at any temperature between 37° and 40° C. We secured one culture of tertian parasites that segmented in four days at the room temperature, in May, of our laboratory at Ancon.

It is highly probable that other red blood cell protozoal parasites of man and animals can be cultivated *in vitro* by this technique or some modification of it. It is also probable that the same principles may be equally applicable to the cultivation of other cell parasites of man and animals. From our observations of malarial plasmodia growing *in vitro* we believe that *in vivo* they can pass from cell to

cell only when a cell is in direct contact with another cell containing a segmenting parasite and then only when the opening for the exit of merozoites occurs opposite the cell to be infected. The substance of malarial plasmodia is very different in consistency from that of red blood cells and therefore they cannot pass through the smallest capillaries like the more yielding fluid-like red blood cell. That the consistency of the protoplasm of the parasite is less yielding than that of the red blood cell is shown by the fact that when a small quantity of a culture containing large parasites is spread over a slide with the end of another slide the parasites are dragged to the end of the spread, though the red blood cells are left behind. Large æstivo-autumnal plasmodia are round or oval; the tertian variety are more or less flattened. If a proper amount of culture containing æstivo-autumnal parasites is placed on a slide and covered with a cover glass, it is found upon studying the specimen under the microscope, while variable pressure is made on the cover glass, that the parasites are not moved, though the red blood cells flow back and forth with great ease. This is due to their round or oval form and firm consistency. Marchiafava and Bignami (6) noted this phenomenon in splenic puncture and autopsy material containing large parasites.

As a result of their unyielding consistency, malarial plasmodia lodge in the capillaries of the body, especially where the current is weakest. Here they remain and develop until they segment. In the meantime other red blood cells are forced against them and if the opening in the infected cell occurs in a favorable location one or more merozoites pass directly into the other cell. Whenever the segmented parasite has become sufficiently broken up it can pass on through the capillary into the circulation where the remaining merozoites are almost instantly destroyed. The debris and pigment are phagocytized by the mononuclear cells of the blood and the endothelial cells of the vessels, and finally are either destroyed or stored in the spleen and other tissues of the body.

We have observed that calcium salts when added to cultures of æstivo-autumnal plasmodia *in vitro* cause hemolysis of the infected red blood cells and possibly also of many non-infected cells. These salts have not the same effect on normal blood. This seems to result

from precipitation of other substances from the serum. The amount of calcium necessary to produce hemolysis is only slightly in excess of the amount present in normal blood and might very possibly be reached by the ingestion of considerable quantities of calcium in drinking water or food. We have herein a suggestion of an explanation of malarial hemoglobinuria. Individual and local susceptibility may be thus explained. What is probably even more striking in this connection is the disappearance of hemoglobinuria, independently of the disappearance of malaria, from localities in which a change from surface lime-containing water to a deep lime-free water supply has been effected.

It is suggested that quinine has no destructive effect upon malarial plasmodia, its effect being possibly to render the red blood cell protecting the parasite more permeable to the all sufficient destructive influence of the serum. If this is true, quinine would affect only the parasites in the circulation and not those lodged in capillaries, which would not be reached by it until they segment. The effect of quinine is defeated by influences such as diet, exertion, etc., which increase the dextrose content of the blood, whereby the permeability of the red blood cells seems to be decreased. These suggestions may possibly lead to a better understanding of the principles involved in the treatment of malaria, and enable us to exchange empiricism for rationalism.

We may have herein an explanation of the manner in which all blood cell parasites of man and animals pass from cell to cell; and it is even possible that the same principles apply to all cell parasites of man and animals.

The size of the capillaries and the amount of blood pressure have an important influence upon the stage at which malarial plasmodia recede from the peripheral circulation. They may be important factors in the production of "cerebral blocking."

SUMMARY.

The asexual cycle of *Plasmodium vivax* and *Plasmodium falciparum* has been cultivated *in vitro* in human blood. The parasites have been grown also in red blood cells in the presence of Locke's solution, free of calcium chlorid and in the presence of ascitic fluid.

The parasites grow within red blood cells and there is no evidence that they can be grown outside of these cells.

The parasites are destroyed in a very few minutes *in vitro* by normal human serum or by all modifications of serum that we have tested. This fact, together with numerous observations of parasites in all stages of growth apparently within red cells, renders untenable the idea of extracorpuscular development.

Leucocytes phagocytize and destroy malarial plasmodia growing *in vitro* only when the parasites escape from their red blood cell capsule or when the latter is perforated or becomes permeable.

Successive generations of *Plasmodium vivax* and *Plasmodium falciparum* have been cultivated *in vitro* by removing the leucocytes from the culture and by transplanting to fresh red blood cells and serum at proper intervals.

The asexual cycle of *Plasmodium vivax* and *Plasmodium falciparum* cultivated *in vitro* does not differ from the same cycle growing *in vivo*.

The sexual cycle has not been cultivated, though we have obtained some evidence of the possibility of its accomplishment.

There can no longer be any doubt that *Plasmodium vivax* and *Plasmodium falciparum* are separate and distinct species. When grown in an identical culture medium and under exactly the same conditions they remain distinct.

In twenty-nine cultures of æstivo-autumnal parasites many forms and sizes have been observed, so that evidence is supplied of the occurrence of different varieties of æstivo-autumnal malarial plasmodia. The so called tertian æstivo-autumnal variety may be seen at the proper stage in all cultures grown from merozoites.

The form and appearance of the same culture of plasmodia may vary greatly under different conditions which are not necessarily destructive to the parasites. Their generation period may vary from thirty hours (æstivo-autumnal) to four days (tertian), as a result of variation in the temperature at which they were cultivated. Sexual parasites grow in the cultures and are more resistant to unfavorable conditions than schizonts, often living several days after the latter die out.

Forms suggesting parthenogenesis have been observed.

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